

DOCKET NO. 3802-126-27 CONT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE APPLICATION OF: De-Chao YU, et al.

Art Unit: 1633

SERIAL NO.: 10/691,045

Examiner: Maria Marvich

FILING DATE: October 21, 2003

FOR: CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN
INTERNAL RIBOSOME ENTRY SITE

AFFIDAVIT OF PRIOR INVENTION UNDER 37 CFR 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, DE-CHAO YU, being duly sworn, depose and state:

1. This Declaration is submitted as evidence that the subject matter claimed in the above-identified application was reduced to practice by the present inventors prior to the publication of Chang et al (WO 99/25860).
2. I understand that the Patent Application was filed on October 21, 2003 with the United States Patent & Trademark Office by Piper Rudnick, and was accorded Serial No. 10/691,045.
3. Andrew Little and I are co-applicants of the above-identified patent application, and the inventors of the subject matter described and claimed therein.
4. Throughout the time period from 1999 to 2001, I was Vice President of Research at Calydon Corporation and was involved in and supervised all projects directed to development of adenovirus vectors, more specifically, Attenuated Replication-Competent Adenovirus ("ARCA") vectors for local and systemic treatment of cancer. One project

of cancer. One project that I supervised was specifically directed to development of ARCA vectors comprising an internal ribosome entry site or "IRES".

5. In my role as Vice President of Research at Calydon Corporation, I supervised the work of Andrew Little who supervised the technical activities performed by Melissa Scott on the project directed to development of ARCA vectors comprising an internal ribosome entry site or "IRES".
6. This Declaration is submitted as evidence that the subject matter claimed in the above-identified application was reduced to practice by the inventors of the instant application prior to the publication of Chang et al. as explained below.
7. I have reviewed the presently pending claims numbered 59-88, as set forth in the amendments dated June 15, 2005 and October 12, 2005.
8. Prior to the publication of Chang et al (WO 99/25860; published May 27, 1999), the inventors of the above-identified application conceived the invention as claimed in at least Claims 59-88 of U.S. Application Serial No. 10/691,045, as noted herein with respect to Exhibits 1-8, which are all dated prior to May 27, 1999. Note that the dates have been redacted from Exhibits 1-8.
9. Page 70-71 of Cell Genesys Notebook # 96 issued to Andrew Little, provides a copy of a memo to me, "DC" from Andrew Little "Andy" (Exhibit 1). The subject matter of the memo is "IRES Strategy" and it is dated December 31, 1998. The memo describes the IRES strategy that was implemented in the experiments summarized in the subsequent pages of notebooks issued to Andrew Little and Melissa Scott. The memo states that the purpose of the project is to regulate expression of both the E1a and E1b genes using a single tissue specific promoter. Both the ECMV and VEGF IRES are referred to in the memo.
10. The memo makes reference to a strategy for cloning a platform plasmid for constructing Ad mutants that express IRESs. The cloning strategy is summarized in a hand written diagram found on page 72 of Genesys Notebook # 96 issued to Andrew Little (Exhibit 2).

11. The experimental details for cloning the platform plasmid and constructing Ad mutants that express IRESs are found on pages 73-117 of Cell Genesys Notebook # 96 issued to Andrew Little (Exhibit 3). The notebook pages describe the construction of two vectors in which the expression of the adenoviral E1a gene is under operative control of either a probasin or CMV promoter and expression of the adenoviral E1b gene is under operative control of an ECMV IRES.
12. Page 117 of Cell Genesys Notebook # 96 states "cloning turned over to Melissa Scott". (Exhibit 3).
13. Page 114 of Cell Genesys Notebook # 99, issued to Melissa Scott states that the goal of the experiment is to regulate expression of both the E1A and the E1B gene using a single tissue specific promoter and that to do so an internal ribosome entry site or (IRES, an internal entry point for initiation of translation by eukaryotic ribosomes) must be cloned between the 2 genes. Both the EMCV and VEG-F IRESs are described. Page 114 further states "Picking up project in the beginning.....where Andy left off" (Exhibit 4).
14. Pages 115-119 of Cell Genesys Notebook # 99, summarize the construction of an adenoviral platform plasmid which has the E1A and E1B genes separated by an EMCV IRES and use of the platform plasmid to make adenoviral vectors which include two different promoters, the probasin ("PB") or cytomegalovirus ("CMV") promoter (Exhibit 5). The experiments detailed by Melissa on pages 116-119 confirm the presence of the IRES in two clones prepared by Andrew Little.
15. The experiments detailed by Melissa Scott on pages 119 and 121 of Cell Genesys Notebook # 99 (Exhibit 6), describe addition of a CMV promoter to the adenoviral platform plasmid. The experiments detailed by Melissa on page 120 and describe addition of a probasin promoter to the adenoviral platform plasmid. (Exhibit 6).
16. Pages 133-134 of Cell Genesys Notebook # 99, issued to Melissa Scott summarize the analysis of constructs and confirmation of the insertion of the CMV and probasin

promoters (Exhibit 7). Five out of eight constructs were demonstrated to have the promoter in the correct orientation (page ***; Exhibit 8).

17. It should be noted that any date blocked out in Exhibits 1-8 were prior to the publication date of the Chang et al. (WO 99/25860), published May 27, 1999.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed at San Francisco, California, this 2nd day of May, 2005⁶

DE-CHAO YU



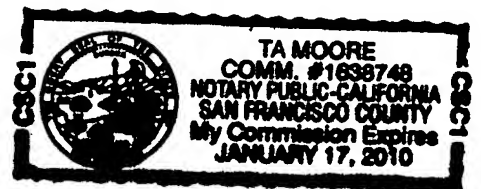
STATE OF California :

COUNTY OF San Francisco:

Before me, a Notary Public for said County, personally appeared DE-CHAO YU, known to me to be the person who executed the foregoing affidavit and acknowledged it to be his act and deed.

Witness my hand and seal this 2nd day of May, 2005⁶





From Page No. V

TO: DC
FROM: ANDY
SUBJECT: IRES STRATEGY
DATE:
CC:

INTRODUCTION

The purpose of the project is to regulate the expression of both the E1a gene and the E1b gene using a single tissue specific promoter. One approach is to engineer both genes to be expressed in one mRNA. To insure efficient translation of the second cistron, an internal ribosome entry site will be cloned between the two genes. After reviewing relevant literature, I determined that two IRESes may be suitable. The first IRES is from encephelomyocarditis virus (EMCV). The EMCV IRES has been well characterized. Researches have delineated the essential bases, and it is commercially available from Novagen (Madison, WI). Others have used the EMCV IRES in gene therapy studies using replication defective retroviruses and adenoviruses. The second IRES selected is from the mouse VEG-F gene. Several genes that regulate cell proliferation and promote cell transformation, like fibroblast growth factor and insulin growth factor, contain IRESes. One hypothesis for why cellular proliferation genes contain IRESes is that cap-dependent translation is not efficient under hypoxic conditions. Huez et al. (1998) recently described a truncated 163 base IRES from a differentially spliced VEG-F mRNA. Their work showed that this IRES was more active than the full length VEG-F IRES and had activity in human cells. A human VEG-F IRES was also recently described. However, it has two independent elements that total 700 bases.

PLATFORM PLASMID CONSTRUCTION

I have attached a strategy for cloning a platform plasmid for constructing Ad mutants that contain IRESes. The first step is to create a deletion in CN306, a plasmid derived from pXC.1 lacking the E1a promoter, that removes the E1B promoter and E1a poly A signal, but leaves the E1a and E1b coding regions intact. This can be accomplished by overlap PCR (see attached). Primers A and C amplify a 288 bp fragment, and primers B and D amplify a 396 bp fragment. Primers C and D were designed to contain 15 b of homology to each other so that the two PCR products could be annealed. This 15 bp region of homology also contains a unique Sal I site that will be

Witnessed & Understood by me,

Invented by

Recorded by

To Page No. 7

used to clone in the IRESes. The Sal I site has been engineered so that IRES inserts will be placed 6 bases (or 2 amino acids) upstream of the E1b translation initiation codon. The overlap product (669 bp) will then be digested with Xba I and Kpn I and cloned into similarly cut CN306. All together, these manipulations delete about 118 bp of endogenous Ad sequence. Once the platform plasmid has been engineered, viruses can be generated by homologous recombination with Ad right hand end plasmids in 293 cells.

EMCV IRES

The EMCV IRES can be cloned from a commercially available plasmid (pCITE-3) using PCR. I suggest using pCITE-3 as a template because the IRES has been modified for optimal performance. Primers E and F amplify a 519 bp IRES fragment with Sal I ends that can be ligated into the platform plasmid described above. The EMCV IRES contains a pyrimidine rich tract of bases near its 3' end that are important for its activity. This pyrimidine tract will be 27 bases (9 amino acids) from the E1b initiation codon.

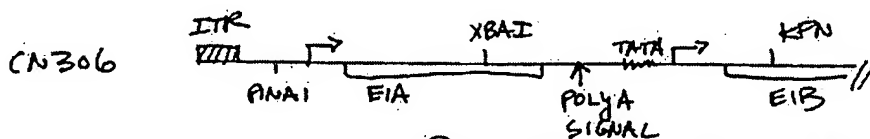
VEG-F IRES

Since the VEG-F IRES is only 163 bases, it may be possible to synthesize it with Sal I linkers. Because this IRES is from an alternatively spliced mRNA and cloning it would require several steps, synthesizing it may save us valuable time. The proposed sequence is attached. Once synthesized, the IRES could be easily cloned into the platform described above.

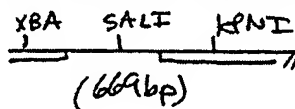
INTELLECTUAL PROPERTY ISSUES

One issue that needs to be considered before beginning the project is if patents applications have been filed on any of these IRESes. If so, do we need to license the IRESes? Or, do we proceed anyway to assess project feasibility before negotiating licensing terms? I suggest that we do a comprehensive intellectual property search.

PLATFORM PLASMID CONSTRUCTION

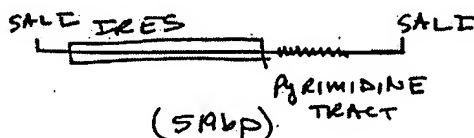


(A) (C) (D) (B)
 GENERATE OVERLAP PRODUCT TO CREATE
 DETECTION OF EIB PROMOTER ETC.



OVERLAP PRODUCT CAN BE CLONED
 DIRECTLY BACK INTO CN306 TO CREATE
 DESIRED PLATFORM PLASMID WITH
 UNIQUE SAI SITE.

EMCV IRES



[Handwritten signature]

VEGF IRES

SENSE PRIMER.

5' ^{SAL I} ACGTAGTCGACAGCGCAGAGGCTTGGGGCAGCCGAGCGGCAGCCA

GGCCCCGGCCCCGGGCCTCGGTTCCAGAAGGGAGAGGAGCCCGCCAAGGCG

CGCAAGAGAGCGGGCTGCCTCGCAGTCCGAGCCGGAGAGGGAGCGCGAGC 1856

CGCGCCGGCCCCGGACGGCCTCCGAAACCA^{SAL I}TGTCGAC ACGTA 3'

ANTISENSE PRIMER

5' ^{SAL I} TACGTGTCGACCATGGTTTCGGAGGCCGTCCGGGGCCGGCGCGGCTCGCG

CTCCCTCTCCGGCTCGGACTGCGAGGCAGCCCGCTCTCTTGCAGCCTTGG 1856

CGGGCTCCTCTCCCTTCTGGAACCGAGGCCCGGGCCGGGGCCTGGCTGCCG

CTCGGCTGCCCAAGCCTCTGCGCTGTCGACTACGT^{SAL I} 3'

THESE TWO OLIGOS WILL BE ANNEALED
AND CLONED INTO UNIQUE SAL I SITE OF
PLATFORM PLASMID.

NOTE - AFTER DISCUSSION WITH
KEYSTONE, I WILL DIVIDE EACH ABOVE
OLIGO IN TWO AND SYNTHESIZE 4 ~906
OLIGOS INSTEAD OF 2 ~1806 OLIGOS.
APPARENTLY, LONGER OLIGOS CAN CONTAIN
DELETIONS/INSERTIONS.

Page No. 73

IRES CLONING OLIGOS

Amplification of EMCV IRES from Novagen pCITE-3a(+) vector

Sense

(96.74.1) GAC GTC GAC ATC GTG TTT TTC AAA GGA A (pCITE sequence 9 to 27) 28

Antisense

(2) GAC GTC GAC TAA TTC CGG TTA TTT TCC A (pCITE sequence 491 to 509) 28

Amplification of E1a/E1b intergenic region

Sense

Primer A

(3) CCT GAG ACG CCC GAC ATC ACC TGT G (Ad sequence 1314 to 1338) 25

Primer D

(4) TGC TGA ATG GTC GAC ATG GAG GCT TGG GAG (Ad sequence 1714 to 1728) 30

Antisense

Primer B

(5) CAC AAC CGC TCT CCA CAG ATG CAT G (Ad sequence 2070 to 2094) 25

Primer C

(6) GTC GAC CAT TCA GCA AAC AAA GGC GTT AAC (Ad sequence 1572 to 1586) 30

Mouse VEG-F oligos to reconstruct SP163

Sense primers



(7) ACG TAG TCG ACA GCG CAG AGG CTT GGG GCA GCC GAG CGG CAG CCA
GGC CCC GGC CCG GGC CTC GGT TCC AGA AGG GAG AGG AGC CCG CCA

90mer

(8) AGG CGC GCA AGA GAG CGG GCT GCC TCG CAG TCC GAG CCG GAG AGG GAG CGC GAG
CCG CGC CGG CCC CGG ACG GCC TCC GAA ACC ATG GTC GAC ACG TA

98mer

Antisense primers

(9) TAC GTG TCG ACC ATG GTT TCG GAG GCC GTC CGG GGC CGG CGC GGC TCG CGC TCC
CTC TCC GGC TCG GAC TGC GAG GCA GCC CGC TCT CTT GCG C

94mer

(10) GCC TTG GCG GGC TCC TCT CCC TTC TGG AAC CGA GGC CCG GGC CGG GGC CTG GCT
GCC GCT CGG CTG CCC CAA GCC TCT GCG CTG TCG ACT ACG T

94mer

notes - underlined regions indicate homology used for overlap PCR
bold regions indicate added restriction sites (e.g. Sall)

↑ ORDER PLACED w/ BIOSOURCE INTERNATIONAL
P.O. 941

Read & Understood by me,

St. M. M.

Invented by

Recorded by

Andrew S. M.

ESTABLISH PCR CONDITIONS FOR PCR AMPLIFYING THE
EIA/EIB INTERGENIC REGION.

<u>RXN</u>	<u>PRIMERS</u>	<u>TEMPLATE</u>	<u>COMMENTS</u>
1	96.74.1/2	PCITE-39	EMCV IRES 519bp
2	96.74.1/2	PCITE-39	
3	96.74.1/2	Ø	
4	96.74.3/6	CN702	Ad EIA SEQUE 288bp
5	96.74.3/6	CN702	
6	96.74.3/6	Ø	
7	96.74.4/5	CN702	Ad EIB SEQUE 396bp
8	96.74.4/5	CN702	
9	96.74.4/5	Ø	

50µl RxN

50µM PRIMER

10mM dNTPs

1µg TEMPLATE

0.5µ DEEP
VENT POL1µl5µl 1µl1µl0.25µlMASTERMIX

50µl BUFF

2.5µl DEEP VENT POL

10µl dNTPs (10mM)

425µl H₂O

94°C X 2'
~~65°C~~
X { 94°C X 45"
65°C X 30" → 55°C 1°/cycle
72°C X 30"

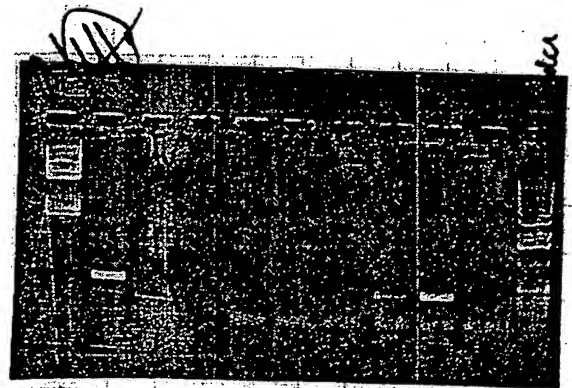
X { 94°C X 45"
55°C X 30"
72°C X 30"

72°C X 2'
4°C HOLDS

POSITIVE AMPLIFICATION
PRODUCT IN LANES

1, 7, 8. AMPLIFICATION
OF AD EIA SEQUENCE

MUST BE REPEATED. PCR MACHINE UN
IS NOT MAKING CONTACT WITH TUBES!



Issued & Understood by me,

M. M.

Invented by

Recorded by

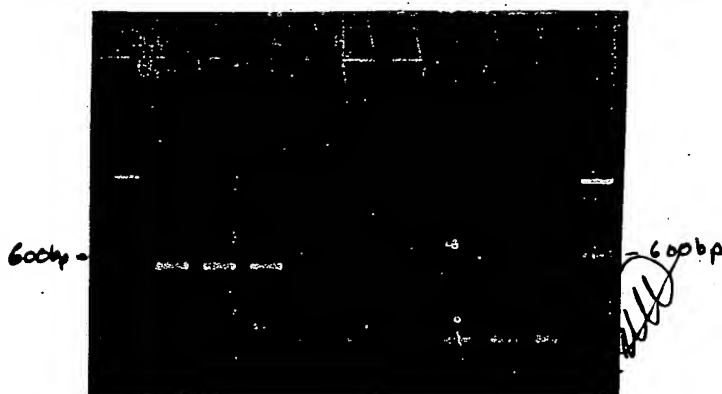
To Page No. 95

IRES CLONING - PLATFORM CONSTRUCTION

96

76

BECAUSE PCR WAS NOT OPTIMAL IN FIRST ATTEMPT
LIKELY BECAUSE LID ON PCR MACHINE IS NOT
MAKING GOOD CONTACT WITH TUBES, I WILL REPEAT
RXNS FOR BETTER YIELD. SAME SETUP AS 96.76.



1% AGAROSE 0.5XTAE

EXPECTED EMCV IRES PREC
AMPLIFIED. EIA/EIB INTERGE
REGION NOT SUCCESSFULLY
AMPLIFIED. I WILL REPEAT
THESE REACTIONS AT 50°
ANNEALING TEMP AND USE
A "HOT START".

1/12/99

REPEAT PCR TO OPTIMIZE CONDITIONS - MODIFY 96.85
BY ALTERING THE ANNEALING TEMPERATURE AND USING
A HOT START.

RXN	PRIMERS	TEMPLATE	SIZE	MASTER
1	96.74.3/6	CN702	288bp	96.76
2	96.74.3/6	CN702	296bp	
3	96.74.3/6	—		
4	96.74.4/5	CN702	396 bp	LEAVE ON POLYMER
5	96.74.4/5	CN702		
6	96.74.4/5	—		

1/12/99

OLIGOS 96.74.7 THROUGH 96.74.10 WERE RECEIVED FROM BIOSOURCE INTERNATIONAL. TO RECONSTRUCT THE MOUSE VEGF IRES DESCRIBED BY HUEB (1998) CALLED SP163 OLIGOS 96.74.7 AND 96.74.10 WILL BE ANNEALED AND OLIGOS 96.74.8 AND 96.74.9 WILL BE ANNEALED.

~~SOME~~

OLIGOS WERE REHYDRATED WITH H_2O AT $1 \mu g/\mu l$. SOME OF 7 AND 10 WERE MIXED AND SOME OF 8 AND 9 WERE MIXED. BOTH MIXTURES WERE DENATURED @ $100^\circ C$ FOR 10' AND ALLOWED TO COOL TO ROOM TEMPERATURE TO ACCOMPLISH ANNEALING.

11 μl OF T4 DNA LIGASE BUFFER WERE ADDED TO EACH TUBE FOLLOWED BY 1 μl NEB PNK (10U/ μl) TO PHOSPHORYLATE OLIGOS. INCUBATE @ $37^\circ C$ X 1H.

HEAT INACTIVATE PNK @ $65^\circ C$ X 20'

~~SUPPLEMENT RXNS WITH~~

ϕ -OH/ $CHCl_3$ EXTRACT, ETOH \downarrow , RESUSPEND IN ^{47 μl} ~~40 μl~~ H_2O . ADD 10 μl DNA LIGASE BUFFER AND 1 μl T4 LIGASE. INCUBATE @ ~~RT~~ ^{RT} ~~45~~ ^{O/N}.

ELECTROPHORESIS
3 μl OF UNLIGATED AND LIGATED OLIGOS TO CONFIRM SUCCESS OF RXNS.



SOME LIGATION APPEARS TO BE OCCURRING. ILLUSTRATED BY THE SMEARING IN LANE 2. I WOULD HAVE EXPECTED BAND @ ~ 190 , HOWEVER. \rightarrow CONCATENERS CAN BE

RESOLVED BY SALT DIGEST To Page No. 89.

IRES CLONING - PLATFORM
CONSTRUCTION

96

Rxn PROFILE

94°C X 2'
94°C X 45"
50°C X 30"
72°C X 30"
4°C HOLD

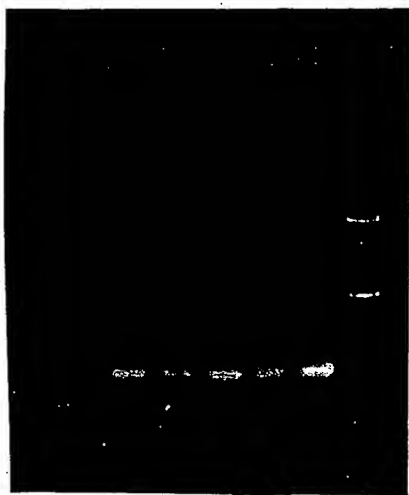
30X

ADD POLYMERASE
AFTER 94°C DENAT
ATION.

ELECTROPHORESIS

1% AGAROSE

0.5X TAE



-600bp

NO PCR AMPLIFICATION SEEN. I NEED TO
CHECK PRIMERS TO MAKE SURE THEY
ARE CORRECT.

1/13/99 REPEAT PCR USING NEW TEMPLATE DNA (CN510-70.166.5)
AND EXPAND LONG POLYMERASE

MASTERMIX #1 (DEEP VENT)

- ✓ 7µl dNTPs
- ✓ 35µl DEEP VENT BUFF.
- ✓ 299µl H₂O

MASTERMIX #2 (EXPAND LONG)

- 7µl dNTPs
- 35µl EXPAND LONG BUFF.
- 298µl H₂O

ADD 0.25µl PRIMERS TO EACH RXN
ADD 0.25µl DEEP VENT OR EXPAND LONG POL
ADD 1µl CN510 (1µg) TO EACH RXN, EXCEPT
CONTROL

11.5/11/99

RXN	PRIMERS	TEMPLATE	MASTERMIX
1	96.74.3/6	CN510	#1
2	96.74.3/6	CN510	
3	96.74.3/6	Ø	
4	96.74.4/5	CN510	#2
5	96.74.4/5	CN510	
6	96.74.4/5	Ø	
7	96.74.3/6	CN510	
8	96.74.3/6	CN510	
9	96.74.3/6	Ø	
10	96.74.4/5	CN510	
11	96.74.4/5	CN510	
12	96.74.4/5	CN510	

XN PROFILE

94°C X 2'

94°C X 45"

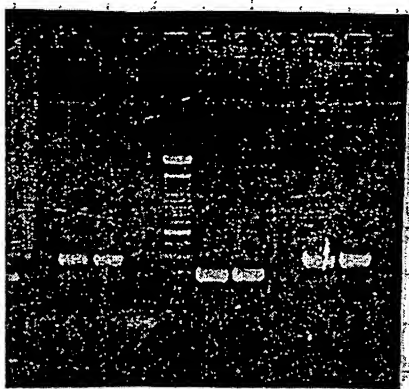
55°C X 30"

72°C X 30"

4°C HOLD

"HOT START"

30X 25X



BOTH PRIMER PAIRS GIVE EXPECTED PCR AMPLIFICATION. PRODUCT (288 + 396 bp). CHANGING DNA TEMPLATE SEEMS TO HAVE MADE THE DIFFERENCE. I WILL GEL PURIFY PRODUCTS FROM DEEP VENT POLYMERASE BECAUSE OF ITS BETTER FIDELITY THAN TAQ.

IRES CLONING - VEGF IRES

96

86

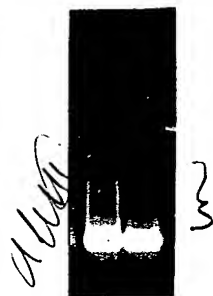
1/13/99

AFTER ALLOWING LIGATION TO PROCEED O/N, I
Φ-OH/CHCl₃ EXTRACTED RXN AND ↓ DNA w/ ETH.

RESUSPEND DNA IN 75μL H₂O. ADD 10μL BSA
ADD 10μL OF 10X SALI BUFFER AND 5μL SALI
(50u).

DIGEST o/n @ 37°C

ELECTROPHORESE LIGATED AND CUT SAMPLES TO
MONITOR PROGRESS OF RXN.



DIGESTION WITH SAL I SEEMS TO HAVE
RESULTED IN LOSS OF HIGHER MOLECULAR
WEIGHT DNA CONCATAMERS. BUT I AM
CONCERNED SINCE MOST OF THE PRODUCT
IS UNLIGATED PRODUCT. I MAY ORDER NEW
PNK AND/OR GEL PURIFY OLIGOS BEFORE
ATTEMPTING LIGATION.

2/22/99

I AM TURNING THIS PROJECT OVER TO MELISSA
SUTT.

[Signature]

[Signature]

STRATEGY

APPRX 200 COLONIES ON EACH LIGATION PLATE. PICK 6 FROM EACH AND GROW MINIPREPS O/N IN 50µl/ml KANAMYCIN.

PURIFY PLASMID DNA USING RPM KIT AVAILABLE FROM BIO101. ELUTE DNA IN 50µl OF A20

DIAGNOSTIC DIGESTS AS FOLLOWS:

PCR BLUNT + PLATFORM 96.91

ACC/NOTI (+) 3766bp
416bp
(-) 3865bp
317bp

XBAI/KPNI (+) 3417bp
601
91
73

(-) 3417bp
593
93
79

PCR BLUNT + EMCV 96.9

HINDIII (+) 3808bp
224bp
(-) 3627bp
405bp

LET DIGEST PROCEED
O/N @ 37°C

ACC/NOTI

15µl DNA
2µl BUFFY
2µl BSA
1µl ENZYME TOTAL
(5µl EACH)

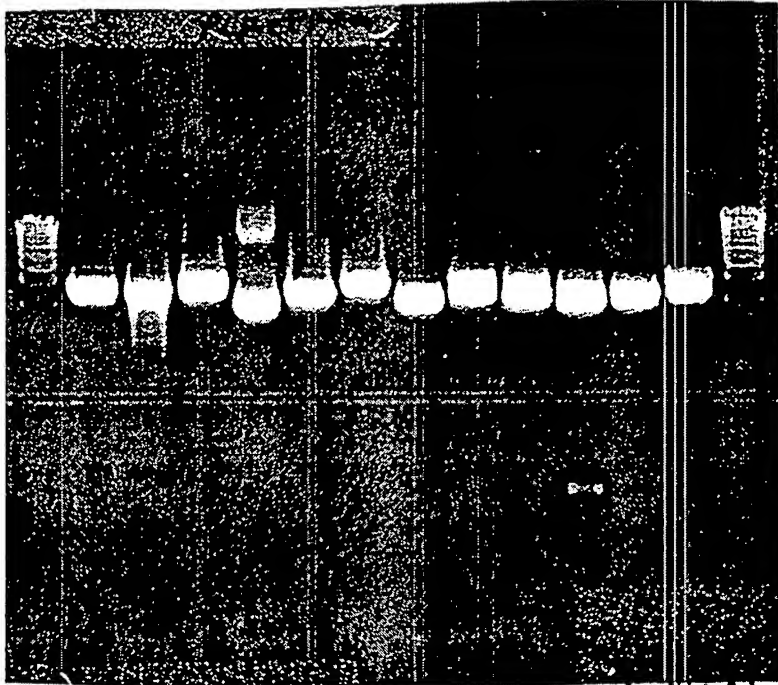
XBAI/KPNI

15µl DNA
2µl BUFFY
2µl H₂O
1µl ENZYME
(5µl EACH)

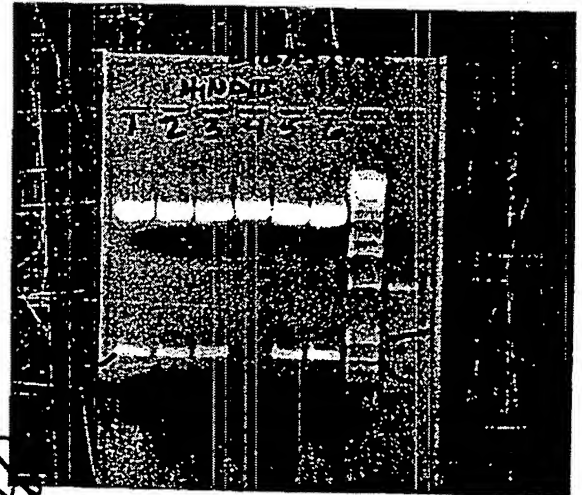
HINDIII

15µl DNA
2µl BUFFY
2µl H₂O
1µl ENZYME
(5µl)

ELECTROPHORESIS 1% AGAROSE



-1.6 kb



ACC/NOTI DIGEST SUGGESTS THAT CLONES 2, 3, 6 ARE RECOMBINANTS IN (-) ORIENTATION. CLONE 1 IS A RECOMBINANT IN THE (+) ORIENTATION. THE XBA/KPN1 DIGEST APPEARS TO BE A PARTIAL DIGEST. A 0.6 kb BAND IS EXPECTED, BUT THE 0.7 kb BAND IS UNEXPECTED. THE DIFFERING INTENSITIES OF THE BAND SUGGESTS THAT THIS IS A PARTIAL DIGEST ALSO. ANOTHER 0.1 kb BAND IS EXPECTED, BUT RAN OFF THE GEL. THE $0.1 \text{ kb} + 0.6 \text{ kb} = 0.7 \text{ kb}$ EXTRA BAND. I WILL GROW UP MAXIS OF CLONES 1, 2 FOR ADDITIONAL CHARACTERIZATION.

CLONES 1, 2, 3, 5, 6 ARE (-) RECOMBINANTS, BUT 4 IS A (+) RECOMBINANT. I WILL GROW UP MAXI OF 1 AND 4 FOR ADDITIONAL CLONING AND CHARACTERIZATION.

done by me
M. M. M.

invented by
M. M. M.

rom Par 100

INOCULATE 200ml CULTURES OF CLONES MENTIONED ON 96.100 WITH LEFTOVER BACTERIAL CULTURES. # (KANAMYCIN 50ug/ml). GROW O/N @ 37°C.

PURIFY PLASMID DNA USING QIAGEN MAXI KIT AS SUGGESTED BY THE MANUFACTURER. 6 DNA w/ ISOPROPANO RESUSPEND IN 500ul H₂O.

ELECTROPHORESE 1ul OF EACH PREP TO DETERMINE CONCENTRATION.



CONCENTRATION OF 96.96 1 #1 = 5ng/ul
 96.96 1 #2 = 200ng/ul
 96.96 2 #1 = 200ng/ul
 96.96 2 #4 = 200ng/ul

DIAGNOSTIC DIGESTS TO RECONFIRM STRUCTURE
 PREP DIGESTS FOR CONTINUING CLONING

KPN1/XBAI

✓ 5ul CLONES 96.96 1 #1 AND #2
 ✓ 2ul BUF 2
 20 UNITS XBAI/KPN1
 ✓ 12ul H₂O
 20ul TOTAL

SALI

✓ 50ul CLONE 96.96 1
 ✓ 20ul SALI BUFF
 4ul SALI (80u)
 20ul BSA
 106ul H₂O
 100ul TOTAL

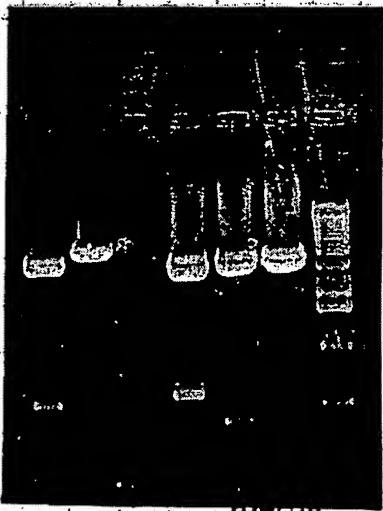
DIGEST STRATEGYHINDIII

✓ 5 μ l DNA CLONE 96.96 2 #1/#4
 ✓ 2 μ l BUFF #2
 ✓ 1 μ l HINDIII (5u)
 ✓ 12 μ l H₂O
 20 μ l TOTAL

SAL I

50 μ l DNA CLONE 96.96
 20 μ l SAL I BUF #1
 20 μ l BSA
 4 μ l SAL I (80u)
 106 μ l H₂O
 200 μ l TOTAL

29. ELECTROPHORESE 5 μ l OF PREP DIGESTS AND 15 μ l OF DIAGNOSTIC DIGESTS TO MONITOR RXNS. 10% AGAROSE.



DIGESTS YIELD EXPECTED RESULTS AS INDICATED ON PHOTOGRAPH. SAL I DIGESTS OF VECTOR (96.96.1 #2) AND INSERT (96.96.2 #1) WERE SUCCESSFUL. NEXT STEP IS TO CIP VECTOR AND THEN GEL PURIFY BOTH VECTOR + INSERT PRIOR TO LIGATION.

96.96.1 #2 = CP 624
96.96.2 #1 = CP 625

O-H/ CHCl₃ EXTRACT VECTOR, PRECIPITATE w/ 3 VOLUMES ETHA. RESUSPEND PELLET IN 90 μ l H₂O. ADD 10 μ l OF NEB BUFFER 3. ADD 2 μ l CIP. INCUBATE RXN FOR 1h @ 37°C. STORE @ -20°C UNTIL PURIFICATION.

From Page No. 102

GEL PURIFY INSERT AND VECTOR - 1% AGAROSE



EXTRACT BANDS, PURIFY DNA
 USING QIAGEN QIAEXTRACT
 ELUTE w/ 10 μ l H_2O .

ELECTROPHORESE 1 μ l 0.8%
 AGAROSE TO DETERMINE YIELD



[VECTOR] = 20 ng/ μ l
 [INSERT] = 5 ng/ μ l

LIGATION SETUP

A
 ✓ 2 μ l VECTOR
 ✓ 2 μ l INSERT
 1 μ l LIGASE
 1 μ l BUFF
 4 μ l H_2O

B
 ✓ 2 μ l VECTOR
 1 μ l LIGASE
 1 μ l BUFF
 6 μ l H_2O

C
 ✓ 2 μ l INSERT
 1 μ l LIGASE
 1 μ l BUFF
 6 μ l H_2O

16 $^{\circ}$ C O/N

DILUTE LIGATIONS 1:10 IN H_2O . TRANSFORM DH10 IS WITH 1 μ l BY ELECTROPORATION. ALLOW TO RECOVER FOR 45' @ 37°C. PLATE ON KAN PLATES INCUBATE @ 37°C O/N.

TMC COLONIES ON PLATE A AND B. DIFFICULT TO DETERMINE IF THERE IS ANY DIFFERENTIAL NO COLONIES ON PLATE C. PICK 12 COLONIES FROM PLATE A. GROW O/N IN KAN/LB.

PURIFY PLASMID DNA FROM MINICULTURES USING BIO 101 RPM KIT (LOT # 2070-600-536-3). ELUTE DNA w/ 50 μ l H_2O .

DIAGNOSTIC DIGESTS w/ HINDIII TO DETERMINE RECOMBINANTS.

EXPECTED DIGESTS:

PLATFORM + IRES (+) ORIENTATION	3.9 kb
	0.8 kb
PLATFORM + IRES (-)	4.1 kb
	0.6 kb

XN
ETUP →

15 μ l DNA
2 μ l NEB 2
3 UNITS HINDIII
3 μ l H_2O

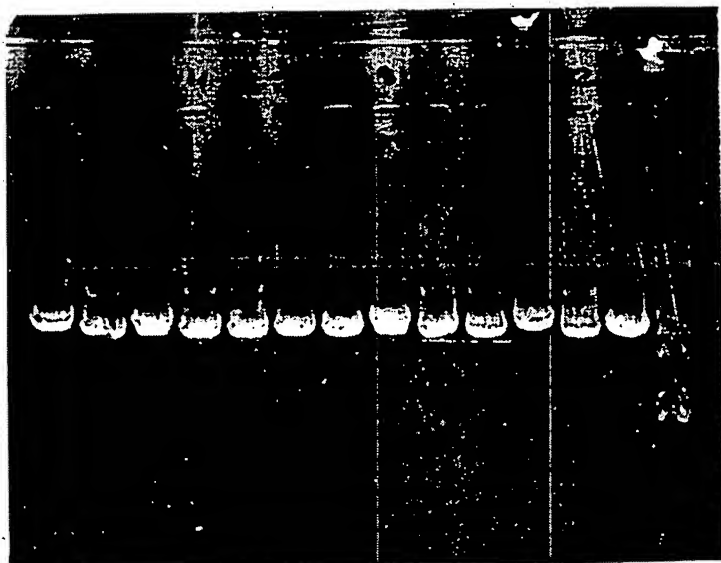
DIGEST FOR 5 hrs @ 37°C

PLATFORM-IRIS CLONING

1041

104

ELECTROPHORESE SAMPLES - 0.6% AGAROSE GEL 70V
0.5X TAE

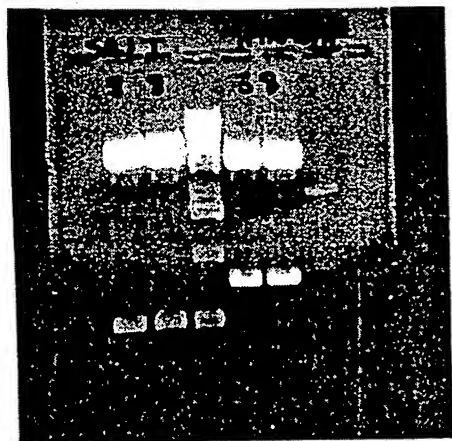


CLONES 3 AND 8 ARE
RECOMBINANTS. HOWEVER
SINCE RESOLUTION IS
POOR, I CANNOT DETER-
MINE ORIENTATION.
I WILL REPEAT THIS
DIGEST TO CONFIRM
CLONES.

VECTOR CONTROL
YIELDED LINEARIZED
PRODUCT 2.4kb AS
EXPECTED.

REDIGEST CLONES 3 &
8 WITH HINDIII (LIKE
AND SALI (96.101) 5h
@ 3:

ELECTROPHORESE - 1%
AGAROSE, 0.5X TAE



SAL I DIGEST, 3 + 8 ARE AS
EXPECTED (INSERT @ 0.5kb). HINDI
DIGEST IS CONSISTENT W/ (+)
ORIENTATION RECOMBINANT. I WILL
STREAK 3 + 8 ON PLATE
AND GROW MAXI PREPS PRIOR
TO SEQUENCING.

96.103.3 = CP626

A. A. C. A. K. H.

IRES CLONING → CN306

96

105

2 200ml CULTURES INOCULATED (50 µg/ml KAN).
GROW O/N @ 37°C

PURIFY 96.103.3 AND 96.103.8 DNA USING
QIAGEN MAXIPREP KIT. ELUTE DNA IN 500 µl H₂O.
STORE @ -20°C UNTIL NEEDED.

BEGIN CLONING OF PLATFORM PLASMID
CONTAINING THE EMCV IRES INTO CN306 BACKBONE

DIGEST 96.103.3 AND CN306 (96.98) WITH KPN I
AND XBA I.

96.103.3 (6P626)
✓ 50 µl DNA (96.107) 10 µg
✓ 20 µl BUFF #2
4 µl XBA (80 U)
✓ 4 µl KPN I (80 U)
✓ 122 µl H₂O

CN306
✓ 50 µl DNA (96.98) -1
✓ 20 µl BUFF #2
4 µl XBA (80 U)
✓ 4 µl KPN I (80 U)
✓ 122 µl H₂O

DIGEST 4h @ 37°C

ELECTROPHORESE 10 μ l OF EACH RXN TO MONITOR PROGRESS OF RXN.



CN306 DIGEST IS COMPLETE.
HOWEVER, 96.103.3 DIGEST PRODUCED UNEXPECTED RESULTS. AFTER INSPECTION OF ORIGINAL CONSTRUCT, I REALIZED THAT 96.103.3 HAS A KPN1 SITE IN THE DREX THAT I HAD NOT ACCOUNTED FOR. EXPECTED BANDS ARE, THEREFORE,

	3.4 kb
	0.6
	0.47
	0.09
	0.07

RESULTS ARE CONSISTENT.

TO GET AROUND THIS PROBLEM, I WILL DO A PARTIAL KPN1 DIGEST AND GEL PURIFY 1 kb BAND.

DIGEST 96.103.3 WITH XBA1 COMPLETELY.
FOLLOW UP WITH PARTIAL KPN1 DIGEST TOMORROW.

130 μ l DNA (26 μ g)
20 μ l BSA
20 μ l BUFF 2
6 μ l XBA1 (120 UNITS)
24 μ l H₂O

DIGEST O/N @ 37°C

M. M. M.

Andrew S. [Signature]

IRIS CLONING

96

108

DIVIDE XBAI DIGEST INTO 3, 65 μ l/TUB
DIGEST EACH TUBE FOR 1H @ 37°C AS
BELOW:

#1
20 UNITS KPN1

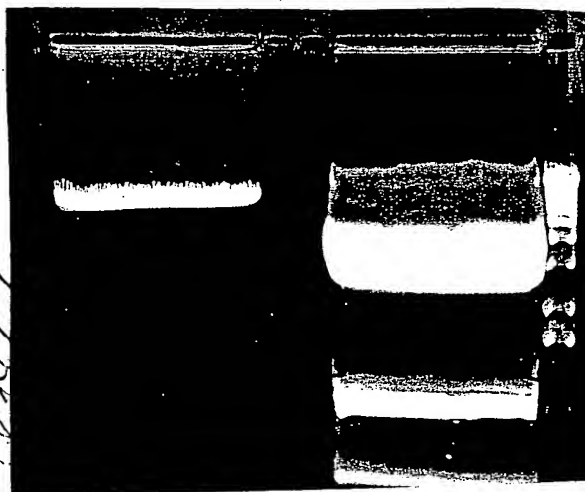
#2
10 UNITS

#3
5 UNITS

AFTER RXN INCUBATION, PLACE TUBES ON ICE
ELECTROPHORESE 5 μ l TO MONITOR RXN
PROGRESS.



PARTIAL DIGEST WAS SUCCESSFUL.
2 kb BAND IS PRESENT. NEXT STEP
IS TO PURIFY BAND BY ELECTROPHORESIS



PURIFY DI
BY QIAEPI
KIT-ELUT
W/ 10 μ l H



DETERMINE [DNA]
PRIOR TO LIGATION

[96.103.3] = 20 ng/l

[~~FAST~~
VECTOR] = 10 ng/l

10.10.11.11.11

Andrew S. [Signature]

109

LIGATION

<u>A</u>	<u>B</u>	<u>C</u>
✓ 5ul VECTOR (50ng/ul)	✓ 1ul IKES INSERT	✓ 5ul VECTOR
✓ 1ul IKES INSERT	✓ 1ul BUFF	✓ 1ul BUFF
✓ 1ul BUFF	✓ 1ul LIGASE	✓ 1ul LIGASE
✓ 1ul LIGASE	✓ 7ul H ₂ O	✓ 3ul H ₂ O
✓ 2ul H ₂ O		

INCUBATE RT X 2H

TRANSFORM LIGATIONS (DILUTED 1:10) DH10B ELECTRO
COMPETENT CELLS. PLATE ON LB/AMP PLATES.
INCUBATE O/N.

TMC COLONIES ON PLATES A AND C. PICK
10 FROM PLATE A FOR MINIPREPES. GROW O/N
@ 37°C.

PURIFY PLASMID DNA USING RPM BIO101 KIT.
ELUTE PLASMID DNA IN 50ul OF H₂O.
CHARACTERIZE RECOMBINANTS WITH KPN1 AND
HINDIII DIGESTS.

Ph.D. Gilman

DIGEST SET UP: SCREEN FOR RECOMBINANTS.

KPN I

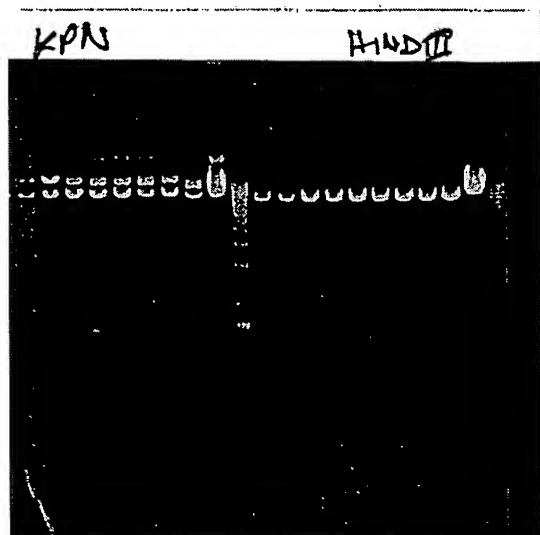
15 μ l DNA
 2 μ l BSA
 2 μ l BUFF2
 1 μ l enzyme (34)

HIND III

15 μ l DNA
 2 μ l GUF2
 1 μ l enzyme (34)
 2 μ l H₂O

DIGEST 4 hrs @ 37°C

ELECTROPHORESIS @ 90V, 0.8% AGAROSE 1hr



EXPECTED BANDS:

HINDIII - 8.3 kb
 1.4 kb

KPN I - 9.3 kb
 0.5 kb

CM306 VECTOR SHOULD BE UNFETTERED
 IN EACH CASE

KPN I DIGEST DOES NOT APPEAR COMPLETE. HOWEVER,
 HIND3 DIGEST INDICATES THAT CLONES 96.115 1, 2, 3, 4, 5, 6,
 MAY BE RECOMBINANTS. ~~2~~ BAND @ 1.5 kb IS
 INDICATIVE. CLONES 1 AND WILL BE MAXIPREPED,
 AND THEIR STRUCTURE WILL BE CONFIRMED WITH
 ADDITIONAL DIGESTS BEFORE CONTINUING.

NOTE: THIS CLONING IS BEING TURNED OVER TO MELISSA
 SCOTT

TITLE EMCV IRES CLONING

Project No. _____

Book No. 96

From Page No. 116

INOCULATE 250ml LB/AMP WITH BACTERIA FROM 96.11
AND 96.115-2 GROW O/N @ 37°C SHAKING.

PURIFY PLASMID DNA USING QIAGEN MAXIPREP KIT
AS SUGGESTED BY MANUFACTURER. ELUTE RESUSPEND
DNA IN 500µl H₂O

CLONING TURNED OVER TO MELISSA SCOTT.

Witnessed & Understood by me,

Invented by

Page No. 96

From Page No. _____

GOAL → to regulate the expression of both the E1A gene and the E gene by using a single tissue specific promoter. To do so an internal ribosome entry site (IRES) must be cloned b/t the 2 g
 ↳ an internal entry point for initiation of translation by euk. ribosomes

2 Potential IRES:

- ① EMCV: encephelomyocarditis virus. Well characterized. Commercially available from Novagen. (450bp) efficient translation. dicistronic message, commonly used in gene therapy.
- ② VEG-F: from mouse, vascular endothelial growth factor. Active in hypoxic environments, 1000nt in length

Advantages of including IRESes in ARCA system

- regulate expression of two Ad genes from a single tissue specific
- regulate expression of one Ad gene and one cytotoxic gene (CD, P450, IL4) from a single TRE
- prevent homologous recombination
- preferential expression of IRES controlled gene in hypoxic cells

Want to design a platform plasmid for constructing Ad mutants that contain IRESes

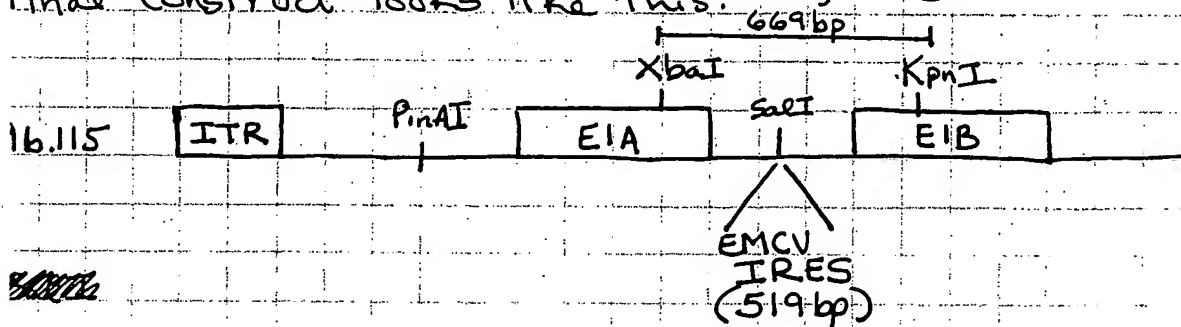
Picking up project in the beginning → where Andy left off.

from Page No. 114

Andy created the following deletion in CN306 (derived from pXC).
↳ lacks the E1a promoter area

Deleted the E1B promoter and E1a poly a signal, but leaves the E1a + E1b coding regions intact.

Final Construct looks like this:



used overlapping PCR to delete E1B promoter poly a signal at the same time introduced a SalI site for insertion of IRESes

Andy → the EMCV IRES was amplified from pCITE3 (Novagen) w/ SalI ends that were used for ligation into the platform plasmid.

The IRES insert will be 6 bases upstream of the E1B translation initiation codon.

The PCR product was then cloned back into CN306 ↑ XbaI / KpnI

The EMCV IRES contains a pyrimidine rich tract of bases near its 3' end that are imp. for its activity. This pyrimidine tract will be 27 bases from the E1b initiation codon.

This is where Andy is at. He would like me to confirm the clone which is the platform plasmid + EMCV IRES. This clone is called 96.115 for now.

Once clone is confirmed want to add 2 different promoters:

- ① PB: probasin promoter, prostate. Can obtain from CP251 w/ PnAI
- ② CMV: cytomegalovirus promoter, expressed 100 fold more. Need to amplify w/ PnAI sites 5' + 3'

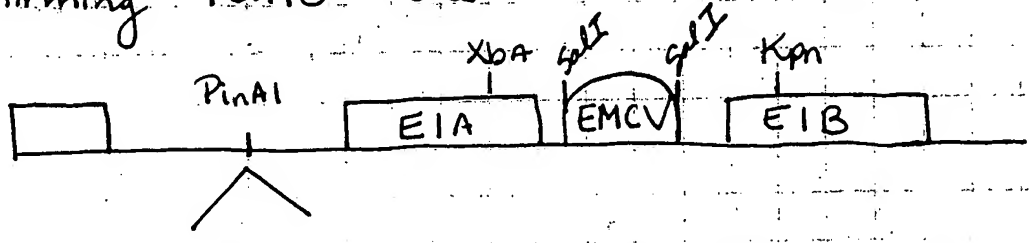
CV787 has prostate specific promoter. CMV active in all cells. Very Active (100 fold more)

To Page No. 11

Witnessed & Understood by me,

Invented by

Confirming 96.115 clone



- ① PB promoter
- ② CMV promoter

CN251 ↑ PinAI fragment
design primers for PCR ↑ PinAI 5' and 3'

1. Digest : Andy has 2 potential clones that need to be confirmed

Sets of Digests:

Xba/KpnI buffer 2 + BSA

CP306 : 8600 bp, 700 bp
96.115 #1 + #2 : 8656 bp, 641, 467

KpnI buffer 1 + BSA

CP306
96.115 #1 + #2

Cla/AvrII buffer 4 + BSA

CP306 9.4 Kb, linear no AvrII
96.115 #1 + #2

PinAI Gibco buffer 4. Doing this digest now in case clones are correct then can proceed right away w/ ligating PB promoter into plasmid construct.

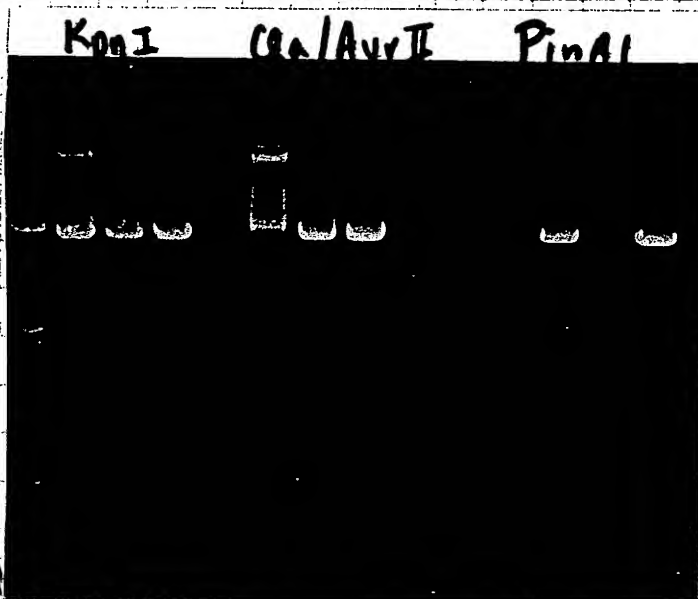
CP251 DNA from DC 1.15 µg/µl (used 2 µl for digestion)

Heather Connors

Heather Connors

From Page No. 116

Xba/Kpn



KpnI

Cla/AvrII

PnaI

Results

Xba/Kpn: ^{CP}306 dropped the correct size fragment → 700bp
 9b.115# 2 is correct. Think #1 is correct too - just difficult to see bands in the ~~the~~ dye bands.

Cla/AvrII: ClaI is not cutting due to dam methylation!!
 Overlooked this. ∴ CP306 is uncut, #1 + #2 are linearized by AvrII

PnaI: #1 + #2 look OK. CP251 is not 1.15µg/µl!

Next: believe #2 looks correct. WANT to be sure.
 Use more DNA + rpt digests: Digest all 3 constr.

① Xba/KpnI buffer 2 + BSA: 10µl DNA

② Hind III 10µl DNA
 Looking for 1.4 and 8.3 Kb band.

CP251: inoculated o/n culture from glycerol stock LB+

From Page No. 11

CP 306

76.115 #1

76.115 #2

Xba/Kpn I

buffer 2

BSA

H3

buffer

2

DNA []10 μ l ($\sim 2 \mu$ g)10 μ l ($\sim 2 \mu$ g)

a) 37°C 9 AM -

Also repeated PinAI digest.

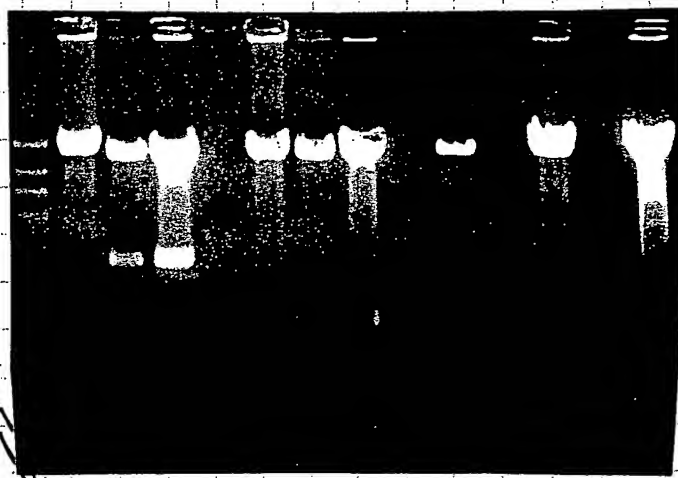
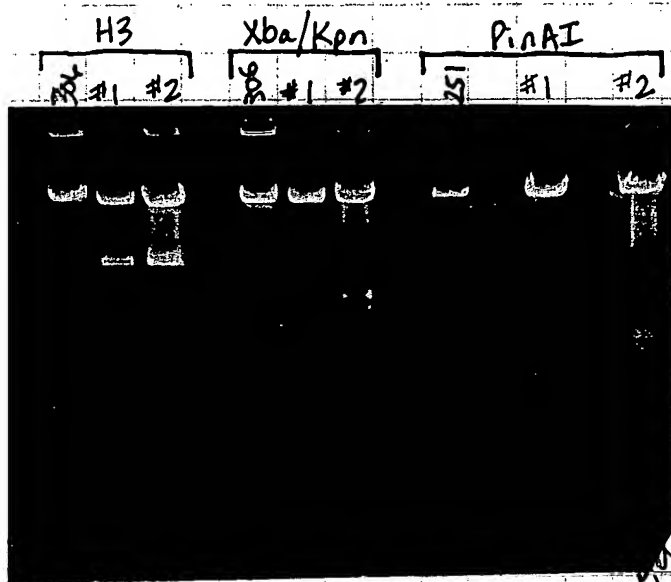
2 mini-preps of CP251. Will ~~add~~ 1 tube to company DNA stock. (C-20)

Digested the following samples w/ PinAI:

CP251 25 μ l DNA ($\sim 2.5 \mu$ g)
 3 μ l Gibco buffer 4
 2 μ l PinAI (10 units)
 30 μ l

96.115 #1 + 2 20 μ l ($\sim 4.0 \mu$ g)
 5 μ l H₂O
 3 μ l Gibco buffer 4
 2 μ l PinAI
 30 μ l

b) 37°C 9:30 -



Results: MARKER looks terrible. Try using Andy's 1Kb ladder.
 HARD to tell size of H3 fragments. Need to rpt digest! (aga)
 Xba/Kpn \rightarrow fragments so faint but both look OK.
 PinAI: Andy thinks maybe there is not enough DNA for 251
 so 500 bp fragment isn't showing up.

To Page No. 11

Witnessed & Understood by me,

Date

Invented by

Heather Connors

Recorded by

William J. Galt

TITLE _____

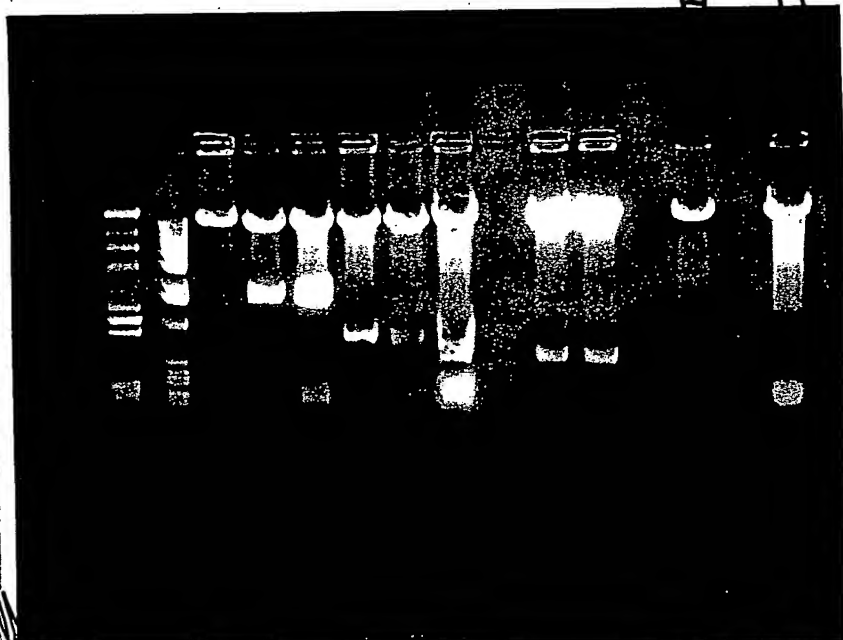
From Page No. 118Next: Do maxi-prep for 251 FINAL Volume 400 μ l

Then set up the following o/n digestions: (maybe digestion will be more com.)

 \uparrow H3 buffer 2: 10 μ l DNA CP306, 96.115 #1 + #2 \uparrow Xba/Kpn buf 2 + BSA: 10 μ l DNA CP306, 96.115 #1 + #2

PinAI: 96.115 #1 + #2: 25 μ l DNA
 3 μ l buffer
 2 μ l enzyme
 30 μ l

CP251: 100 μ l DNA (~20 μ g)
 12 μ l buffer
 8 μ l PinAI (40 Units)
 120 μ l



96.115 #1 + #2 look correct

CP627: 96.115 #2

★ see page 135

@ Purified: CP251 500bp fr
 96.115 \uparrow PinAI #1 9Kb
 #2 9Kb

CIP Treated Both #1 and #2
 (used same protocol as on p. 35)

17 μ l DNA (gel purified)
 2 μ l buffer
 1 μ l CIP (Boehringer)
 20 μ l

@ 37°C 1h

Added 0.2 μ l of .5M EDTA

@ 75°C 10 min

Phenol / CHCl₃EtOH ppt. (15 μ l)Resuspend in 15 μ l H₂O

Witnessed & Understood by me,

Heather Corneau

Invented by

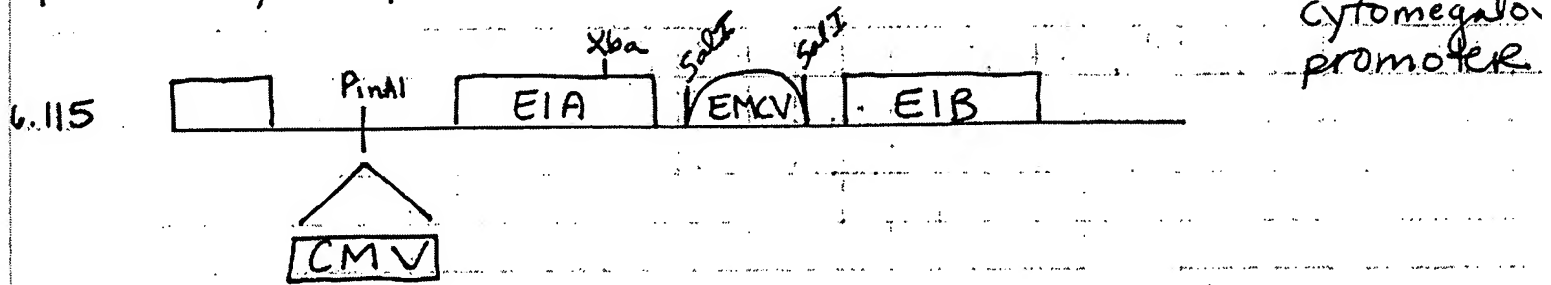
Records Heather Corneau

To Page #

om Page No.

Goal → also want to add CMV promoter to platform construct...
PCR promoter + clone into PinAI site of 96.115

CMV promoter will act as a control → the probasin promoter is specific to the prostate. CMV is active in many different types of cells. Can use these 2 for comparison → look at specificity of probasin construct.



Primer Design

2/23/99

99.120.1 FW Primer. PinAI (Anneals to 27-41 in pCMVB, Clontech) $T_m = 40C$

5'- ACG TAC ACC GGT CGT TAC ATA ACT TAC -3'

99.120.2 RV Primer. PinAI (Anneals to 535-548 in pCMVB, Clontech) $T_m = 42C$

5'- CTA GCA ACC GGT CGG TTC ACT AAA CG-3'

Min

To Page No. 1

Witnessed & Understood by me,

Teacher Cornaro

Invented by

Recorded by

Min

Project No. _____
Book No. 99

TITLE Ligation of PB into Platform Construct

From Page No. 119

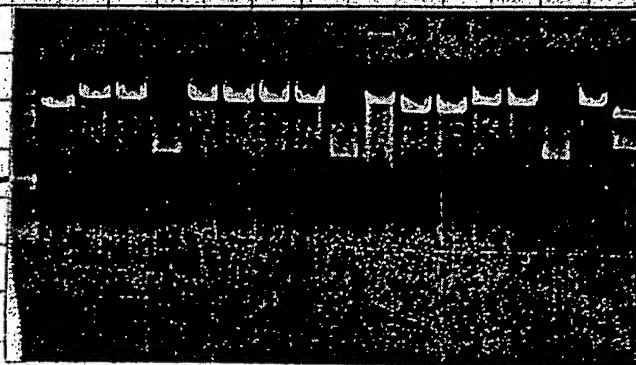
Ligation Platform Construct + Probasin promoter
4 μ l vector (CIP treated 96.115)
6 μ l fragment (CP251 + P_{MAI})
12 μ l buffer
8 μ l ligase
2.0 μ l
@ 16°C o/n

Transform JM109 180 μ l + 12 μ l ligation rxn LB + Amp

26. Colonies w/ both constructs
Stored plates at 4°C → pick colonies 2/28/99

8. Picked 16 colonies (96.115 #2 + PB promoter)
4mg LB + Amp @ 37°C o/n

Mini-Preps
R. Digest NruI / BclI Expected fragment size: 6334,
~~6334~~ → unique in PB promoter
10 μ l DNA
2 μ l buffer 7 Gibco
1 μ l NruI
6 μ l H₂O
19 μ l @ 37°C 1hr
heat inactivate @ 65°C 20min
add 1 μ l BclI @ 50°C 1hr



Run gel longer → see page 129

Page No. 132

10 Transformation very efficient - maybe self-ligation.
Check 9 transformants for each clone.

Inoculated 4ml LB + Amp @ 37°C O/N

① #1-9 CMV + CP306

② #10-18 PB + CP306

Mini-preps

R. Digest

↑ PstAI buffer 4 Gibco

20 µl DNA

2.5 µl Gibco 4

2.5 µl PstAI (12.5 U)

25.0 µl

@ 37°C

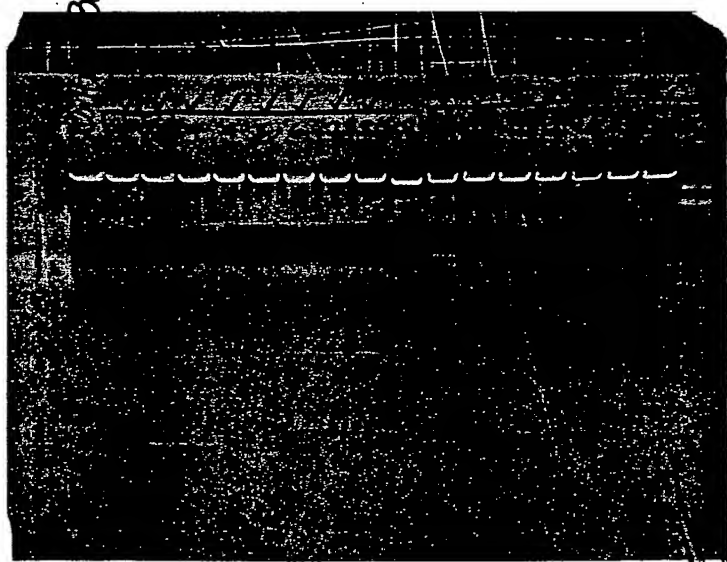
10:30 - 1:30 pm

#18 did not fit on gel!

Results:

CMV 8/9 correct

PB 6/8 correct



Page No. _____

Witnessed & Understood by me,

Cathy Connors

Invented by

Recorded by

[Signature]

Page No. 133

Confirm Orientation of the following clones:

~~CP1506~~ + CMV # 1, 4, 5, 7, 17

~~CP1506~~ + PB # 2, 3, 5, 6, 7, 8, 13, 14, 16

same digests as before for screening: (p130)

CMV Constructs: Sst/H3 buffer 2

PB Constructs: ~~MS~~ H3 buffer 2 + ~~BSA~~ ^{MS}

@ 37^{10AM} ~~10AM~~ - 4pm

15 μ l DNA

1 μ l enzyme

1 μ l enzyme

2 μ l buffer

20 μ l

1 \uparrow Sst/H3 buffer 2

ROL 96.115

5480

2003

1030

841

410

Negative Orientation

4290

2003

1871

1718

410

Positive

4780

2003

1228

1030

841

410

\uparrow H3 buffer 2

Negative Orientation

7603

1440

1219

Positive Orientation

7155

1677

1440

CONTROL

8362

1440

To Page No. 135

Read & Understood by me,

Arthur Connors

Invented by

Recorded

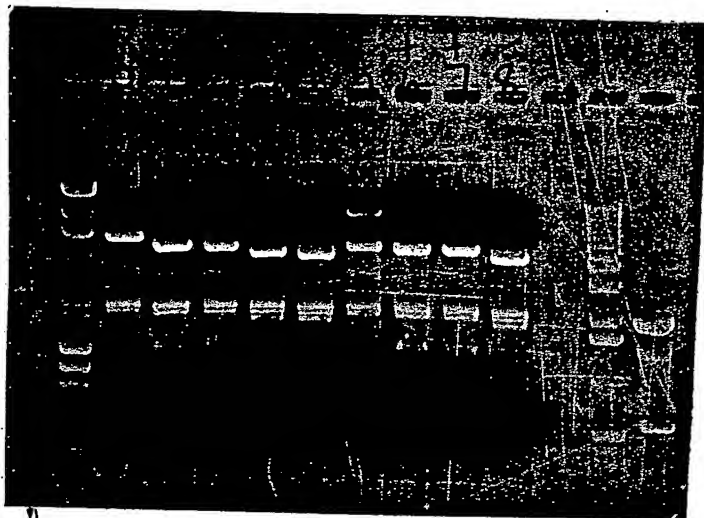
W. H. Smith

TITLE CP 632 , CP 633

Project No. _____

Book No. _____

From Page No. _____



Results: 5/8 CORRECT orientat

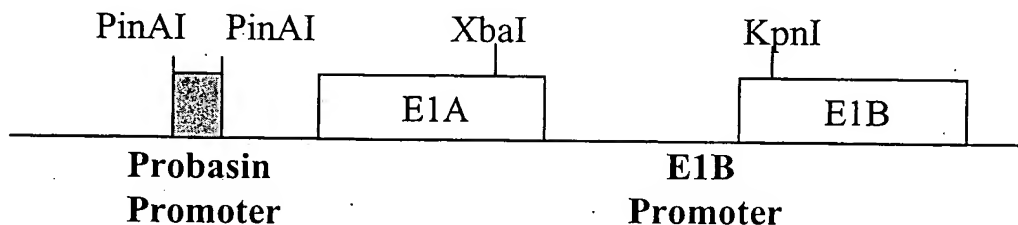
#1, 2, 5, 6, 7

CP633

Control Constructs

CP632: CP306 + Probasin promoter obtained from CP251 digested with PinAI. Control Protein- **E1B** promoter and E1A poly A signal present, no IRES.

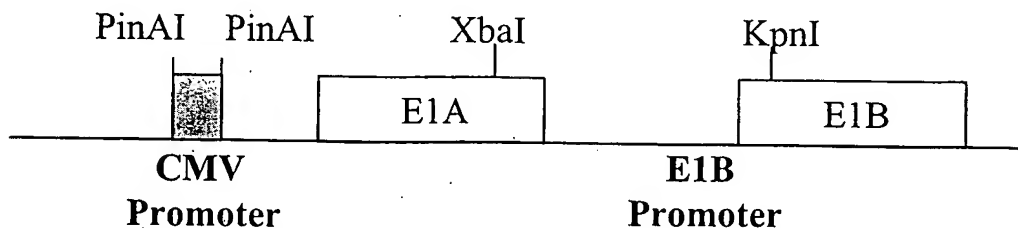
#10
99.137



Glycerol
Stocks
Prepared
3/16

CP633: CP306 + CMV promoter amplified from pCMVbeta (Clontech) with PinAI 5' and 3' ends for ligation. Control Protein- **E1B** promoter and E1A poly A signal present, no IRES.

#1
99.139



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